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FILE NO.A31304-B-A-F 069906.0105
PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Mitchell et al
Serial No. : 10/076,248 Examiner : TBD
Filed : February 12, 2002 Group Art Unit: TBD
For : METHODS AND COMPOSITIONS FOR USE IN SPLICEOSOME
MEDIATED RNA TRANS-SPLICING

SUBMISSION OF SEQUENCE LISTING

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July 22, 2002
Date of Deposit

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Carmella L. Stephens
Signature

July 19, 2002
Date of Signature

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In accordance with 37 C.F.R. §1.821-1.825, Applicants submit herewith an initial Sequence Listing in computer and paper form.

IN THE SPECIFICATION

Please **amend** paragraph 22 on page 11 as follows:

Figure 4. Diagram and important structural elements of double *trans*-splicing PTM7. The double splicing PTM7 has both 3' and 5' functional splice sites as well as binding domains (SEQ ID NOS:9-15).

Please **amend** paragraph 25 on page 11 as follows:

Fig. 6C. The accuracy of double *trans*-splicing of synthetic PTM RNA in 293T cells was verified by sequencing the spliced RNA produced by RT-PCR (SEQ ID NOS:16 and 17).

Please **amend** paragraph 41 on page 18 as follows:

A nucleotide sequence encoding a translatable protein capable of producing an effect, such as cell death, or alternatively, one that restores a missing function or acts as a marker, is included in the PTM of the invention. For example, the nucleotide sequence can include those sequences encoding gene products missing or altered in known genetic diseases. Alternatively, the nucleotide sequences can encode marker proteins or peptides which may be used to identify or image cells. In yet another embodiment of the invention nucleotide sequences encoding affinity tags such as, HIS tags (6 consecutive histidine residues) (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-8976), the C-terminus of glutathione-S-transferase (GST) (Smith and Johnson, 1986, Proc. Natl. Acad. Sci. USA 83:8703--8707) (Pharmacia) or FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) (SEQ ID NO:1) (Eastman Kodak/IBI, Rochester, NY) can be included in PTM molecules for use in affinity purification. The use of PTMs containing such nucleotide sequences results in the production of a chimeric RNA encoding a fusion protein containing peptide sequences normally expressed in a cell linked to the peptide affinity tag. The affinity tag provides a method for the rapid purification and identification of

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peptide sequences expressed in the cell. In a preferred embodiment the nucleotide sequences may encode toxins or other proteins which provide some function which enhances the susceptibility of the cells to subsequent treatments, such as radiation or chemotherapy.

Please **amend** paragraph 60 on page 25 as follows:

Alternatively, synthetic PTMs can be generated by *in vitro* transcription of DNA sequences encoding the PTM of interest. Such DNA sequences can be incorporated into a wide variety of vectors downstream from suitable RNA polymerase promoters such as the T7, SP6, or T3 polymerase promoters. Consensus RNA polymerase promoter sequences include the following:

T7: TAATACGACTCACTATAGGGGAGA (SEQ ID NO:2)

SP6: ATTTAGGTGACACTATAGAAAGNG (SEQ IDNO:3)

T3: AATTAACCCTCACTAAAGGGGAGA. (SEQ ID NO:4)

The base in bold is the first base incorporated into RNA during transcription. The underline indicates the minimum sequence required for efficient transcription.

Please **amend** paragraph 92 beginning on page 37 and ending on page 38 as follows:

Total cell RNA (2.5 µg) from the transfections was converted to cDNA using the MMLV reverse transcriptase enzyme (Promega) in a 25 µl reaction following the manufacturers protocol with the addition of 50 units RNase Inhibitor (Life Technologies) and 200 ng Lac-6R gene specific primer:

(5'-CTAGGCGGCCGCCTGCTGGTGTGTTTGCTTCC). (SEQ ID NO: 5)

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cDNA synthesis reactions were incubated at 42°C for 60 min followed by incubation at 95°C for 5 min. This cDNA template was used for PCR reactions. PCR amplifications were performed using 100 ng primers and 1 µl template (RT reaction) per 50 µl PCR reaction. A typical reaction contained ~25 ng of cDNA template, 100 ng of primers (common to cis- and trans-spliced products) (KI-1F, 5'-GTTTCGCTAAATACTGGCAGG and, Lac-6R, 5'-CTAGGCGGCCGCCTGCTGGTGTGTTTGCTTCC) (SEQ ID NOS:6 and 7) 1X REDTaq PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM MgCl₂ and 0.1% gelatin), 200 µM dNTPs and 1.5 units of REDTaq DNA polymerase (Sigma, Saint Louis, Missouri). PCR reactions were performed with an initial pre-heating at 94°C for 2 min 30 sec followed by 20 cycles of 94°C for 30 sec (denaturation), 60°C for 36 sec (annealing) and 72°C for 1 min (extension) followed by a final extension at 72°C for 7 min. The PCR products were then digested with Sph I and Dde I restriction endonucleases, which specifically cleaves cis-spliced product. Trans-spliced product was isolated using Lac-21 (has biotin at the 5' end) as a hybridization probe. The purified trans-spliced product was subjected to a 2nd round of nested PCR using primers KI-2F (5'-CTGGCAGGCGTTTCGTCAG) (SEQ ID NO: 8) and Lac-6R. Authenticity of the trans-spliced product was further confirmed by diagnostic digestion with Pvu I restriction enzyme which specifically cleaves the trans-spliced product.

Please **amend** paragraph 94 on beginning on page 38 and ending on page 39 as follows:

Using *in vitro* synthesized PTM RNA as genetic material, the results described herein demonstrated the accurate trans-splicing of double splicing exogenously synthesized PTM RNAs (DSPTM7, CFTR targeted; DSPTM19, βHCG targeted) into pre-mRNA target (Fig. 2)

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both in co-transfection assays (293T cells) as well as in cells that express double splicing lacZ pre-mRNA target from an integrated genomic locus. For this purpose, DSPTM6, DSPTM7, DSPTM18 and DSPTM19 (capped and uncapped) (Fig. 3) RNAs were exogenously synthesized using bacteriophage T7 RNA polymerase *in vitro*, gel purified and used for transfections. 48 hrs post-transfection, total cellular RNA was isolated and analyzed by RT-PCR (as described above). As shown in Figure 6A, both DSPTM6 and DSPTM7 produced the expected *trans*-spliced 220 bp RT-PCR product in 293T cells (upper panel). The authenticity of this product was confirmed by diagnostic digestion using Sph I, which cuts the cis-spliced product specifically (lower panel, lanes 1 and 2) and Pvu I that cuts the *trans*-spliced product specifically (lower panel, lanes 4 and 5). To confirm that *trans*-splicing between DSPTM7 and DSCFT1.6 pre-mRNA is precise, RT-PCR amplified product was excised, re-amplified using KI-2F and Lac6R primers and sequenced directly using KI-2F or Lac-6R primers. As shown in Figure 6C, *trans*-splicing occurred exactly at the predicted splice sites, confirming the precise internal exon substitution by the double *trans*-splicing events (SEQ ID NOS:16 and 17).

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REMARKS

Applicants submit herewith an initial Sequence Listing in computer and paper form, in accordance with 37 C.F.R. §1.821-1.825. The content of the paper and computer readable copies of the Sequence Listing submitted in accordance with 37 C.F.R. §1.821(c) and (e) are the same and do not include new matter.

Attached hereto is a marked-up version of the changes made to the specification. The attached page is captioned "VERSION WITH MARKINGS TO SHOW CHANGES MADE" and is only included for the Examiner's convenience. Should any discrepancies be discovered, the version presented in the preceding "IN THE SPECIFICATION" section shall take precedence.

CONCLUSION

Please charge any additional fees associated with this filing or credit any overpayment to Deposit Account No. 02-4377. Two copies of this paper are enclosed.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADEIN THE SPECIFICATION

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buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM MgCl₂ and 0.1% gelatin), 200 µM

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reactions were performed with an initial pre-heating at 94°C for 2 min 30 sec followed by 20

cycles of 94°C for 30 sec (denaturation), 60°C for 36 sec (annealing) and 72°C for 1 min

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